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(54) Title: INHIBITORS OF DIPEPTIDYL-AMINOPEPTIDASE TYPE IV

(57) Abstract

Inhibitors of Dipeptidyl-Aminopeptidase Type IV having the following general formula: X-Pro-Y-boroPro, where X and Y are chosen from any amino acid (including proline).

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Inhibitors of Dipeptidyl-Aminopeptidase Type IV

Background of the Invention

This invention relates to inhibitors of the amino peptidase activity of dipeptidyl amino peptidase type IV (DP IV).

DP IV is a serine protease present in many microbes, mammalian cells and tissues, for example, renal tubule cells, intestinal epithelium, and blood plasma. It is also present on the surface of CD-4+ and some CD-8+ T-cells, and in low amounts in the central nervous system. It is thought to be involved in the regulation of the immune response; occurrence of DP IV on a cell surface is associated with the ability of cells to produce interleukin 2 (IL-2). DP IV is also referred to as DAP IV or DPP IV; it is assigned EC number 3.4.14.5.

Three different inhibitors of DP IV are known. One of these is a suicide inhibitor: N-Ala-Pro-O-(nitrobenzyl-) hydroxylamine. (The standard three letter amino acid codes are used in this application; O represents oxygen.) Another is a competitive inhibitor: e-(4-nitro)benzoxycarbonyl-Lys-Pro. The third is a polyclonal rabbit anti-porcine kidney DP IV immunoglobulin.

Summary of the Invention

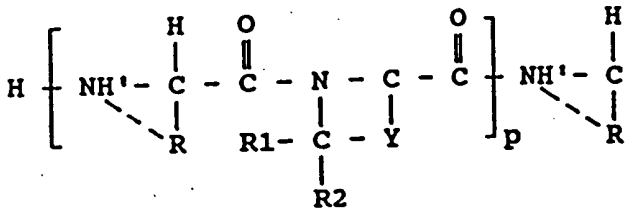
The enzymatic activity of DP IV involves cleaving of a dipeptide from the free amino terminus of a polypeptide. DP IV has a preference for cleaving after a proline, i.e., a proline in the penultimate position from the amino terminus. A free amino terminus is required; thus, DP IV is a postproline cleaving enzyme with a specificity for removing an N-terminal W-Pro dipeptide from a polypeptide (where W can be any amino acid, including proline). DP IV

also will remove a W'-Ala dipeptide from an amino terminus of a polypeptide when W' is an amino acid with a bulky side group, e.g., tyrosine.

This invention concerns provision of potent inhibitors of the enzymatic activity of DP IV. Generally, an α-amino boronic acid analog of proline (boroPro is used to designate one such analog which has the carboxyl group of proline replaced with a $B(OH)_2$ group, where $(OH)_2$ represents two hydrogen groups and B represents boron) is bonded to an amino acid to form a dipeptide with boroPro as the C-terminal residue. These dipeptide prolyl- boronic acids are potent and highly specific inhibitors of DP IV activity and have Ki values in the nanomolar range.

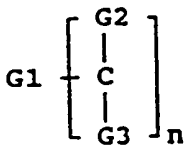
Dipeptides having the boroPro moiety are unstable; thus, we have designed inhibitors having at least two other amino acids. Generally, the structure of these inhibitors is X-Pro-Y-boroPro where X and Y are chosen from any amino acid (including proline). This tetrapeptide may be lengthened at its N-terminus by addition of one or more dipeptides, each dipeptide having the general formula Z-Pro or Z-ala, where each Z independently is any amino acid (including proline). This general structure is defined in more detail below. These inhibitors function as inhibitors of DP IV because each dipeptide portion is a substrate for DP IV and the final product of the reaction of an inhibitor with DP IV is the dipeptide inhibitor Y-boroPro. The amino terminus of these inhibitors must not be blocked or they lose their inhibitory capacity for DP IV, since DP IV cannot cleave a dipeptide from a blocked N-terminal polypeptide.

Thus, in a first aspect, the invention features an inhibitory compound having the structure: Group I - Group II. Group I has the structure:

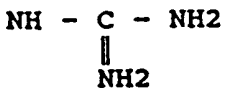


where H represents a hydrogen; C represents a carbon; O represents an oxygen; N represents a nitogen; each R, independently, is chosen from the group consisting of the R groups of an amino acid, including proline; each broken line, independently, represents a bond to an H or a bond to one R group, and each H' represents that bond or a hydrogen; and p is an integer between 0 and 4 inclusive.

Alternatively Group I has the structure:



where n is between 0 and 3 inclusive, each G2 and G3 independently is H or C1 - 3 (one to three carbon atoms) alkyl, G1 is NH3 (H3 represents three hydrogens),

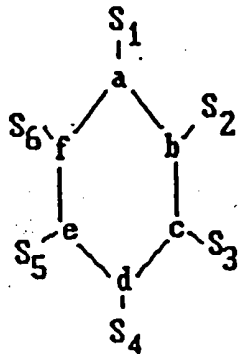


(H2 represents two hydrogens), or

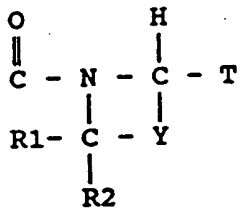
NG4, where G4 is 

where G5 and G6 can be NH, H, or C1 - 3 alkyl or alkenyl with one or more carbons substituted with a nitrogen. G1 bears a charge, and G1 and Group II do not form a covalently

bonded ring structure at pH 7.0. Group I may also have the structure:



where one or two of the a, b, c, d, e, and f group is N, and the rest are C, and each S1 - S6 independently is H or C1 - C3 alkyl. Group I may also include a five membered unsaturated ring having two nitrogen atoms, e.g., an imidazole ring. Group II has the structure:

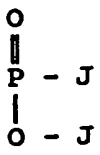


where T is a group of the formula:

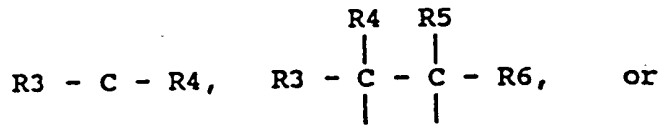
- B - D1, where each D1 and D2, independently, is a hydroxyl group or a group which is capable of being hydrolysed to a hydroxyl group in aqueous solution at physiological pH; a group of the formula:

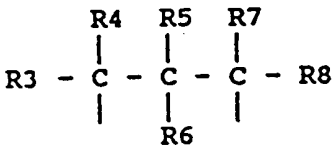
where G is either H, fluorine (F) or an alkyl group containing 1 to 20 carbon atoms and optional heteroatoms

which can be N, S (sulfur), or O; or a phosphonate group of the formula:



where each J, independently, is O-alkyl, N-alkyl, or alkyl. Each O-alkyl, N-alkyl or alkyl includes 1 - 20 carbon atoms and, optionally, heteroatoms which can be N, S, or O. T is generally able to form a complex with the catalytic site of a DP IV.

Y is  , or



and each R1, R2, R3, R4, R5, R6, R7, and R8, separately is a group which does not significantly interfere with site specific recognition of the inhibitory compound by DP IV, and allows a complex to be formed with DP IV.

In preferred embodiments, T is a boronate group, a phosphonate group or a trifluoroalkyl ketone group; each R1-R8 is H; each R1 and R2 is H, and each Y is the CH_2-CH_2; each R is independently chosen from the R group of proline and alanine; the inhibitory compound has a binding or dissociation constant to DP IV of at least 10^{-9}M, 10^{-8}M or even 10^{-7}M; the inhibitory compound is admixed with a pnarmaceutically acceptable carrier substance; and each D1

and D2 is, independently, F, or D1 and D2 together are a ring containing 1 to 20 carbon atoms, and optionally heteroatoms which can be N, S, or O.

In a second aspect, the invention features a method for inhibiting the enzymatic activity of DP IV in a bacterium or mammal. The method includes administering to the mammal an effective amount of an inhibitory compound described above. Most preferably, the amount of compound administered is between 1 - 500 mg/kilogram of animal treated/day.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments, and from the claims.

Description of the Preferred Embodiments

The drawings will first be briefly described.

Drawings

Figure 1 is a diagrammatic representation of the synthesis of a boro proline compound; and

Figure 2 is a diagrammatic representation of several embodiments of the invention.

Structure

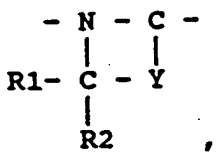
The inhibitory compounds of the invention have the general structure recited in the Summary of the Invention above. Examples of preferred structures are those referred to as preferred embodiments above.

The structure of the inhibitory compounds is such that at least a portion of the amino acid sequence near the cleavage site of a DP IV substrate is duplicated, or nearly duplicated. This duplication is in part responsible for the ability of the inhibitory compounds to inhibit DP IV, by a mechanism thought to involve competitive inhibition between a DP IV inhibitory compound or DP IV cleavage product of the inhibitory compound, and the actual DP IV substrate.

The choice of amino acid sequence affects the inhibitory activity of the inhibitory compound, and its specificity. Peptide fragments can be synthesized and then tested to determine their efficacy as inhibitors, using standard techniques. Specificity is determined in a similar fashion, by testing the inhibitory effect of a particular inhibitory compound on the enzyme activity. The inhibitory compounds preferably inhibit the enzymatic activity of DP IV and do not inhibit enzymes necessary for normal cell functions.

The inhibitory compounds include a group (T) which causes the inhibitory compound to complex with DP IV, not only in a competitive fashion, but in a chemically reactive manner to form a strong bond between the inhibitory compound and DP IV. This group thus acts to bind the inhibitory compound to DP IV, and increases the inhibitory binding constant (Ki) of the inhibitory compound. Examples of such groups include boronates, fluoroalkyl ketones and phosphoramidates (of the formulae given in the Summary above). These groups are covalently bonded to the prolyl residue of the compound, as in the above formula.

The proline or proline analog, represented by



above, is chosen so that it mimics the structure of proline recognized by the active site of DP IV. It can be modified by providing R1 and R2 groups which do not interfere significantly with this recognition, and thus do not significantly affect the Ki of the compound. Thus, one or more hydroxyl groups can be substituted to form hydroxy-

proline, and methyl or sugar moieties may be linked to these groups. One skilled in the art will recognize that these groups are not critical in this invention and that a large choice of substituents are acceptable for R1 and R2.

Synthesis

Synthesis of boroProline

Referring to Figure 1, the starting compound I is prepared essentially by the procedure of Matteson et al., 3 Organometallics 1284, 1984, except that a pinacol ester is substituted for the pinanediol ester. Similar compounds such as boropipecolic acid and 2-azetodine boronic acid can be prepared by making the appropriate selection of starting material to yield the pentyl and propyl analogs of compound I. Further, Cl can be substituted for Br in the formula, and other diol protecting groups can be substituted for pinacol in the formula, e.g., 2,3-butanediol and alpha-pinanediol.

Compound II is prepared by reacting compound I with $[(CH_3)O_3Si]_2N$-Li+. In this reaction hexamethyldisilazane is dissolved in tetrahydrofuran and an equivalent of n-butyllithium added at -78°C. After warming to room temperature (20°C) and cooling to -78°C an equivalent of compound I is added in tetrahydrofuran. The mixture is allowed to slowly come to room temperature and to stir overnight. The alpha-bis[trimethylsilane]-protected amine is isolated by evaporating solvent and adding hexane under anhydrous conditions. Insoluble residue is removed by filtration under a nitrogen blanket, yielding a hexane solution of compound II.

Compound III, the N-trimethysilyl protected form of boroProline is obtained by the thermal cyclization of compound II during the distillation process in which

compound II is heated to 100-150°C and distillate is collected which boils 66-62°C at 0.06-0.10 mm pressure.

Compound IV, boroProline-pinacol hydrogen chloride, is obtained by treatment of compound III with HCl:dioxane. Excess HCl and by-products are removed by trituration with ether. The final product is obtained in a high degree of purity by recrystallization from ethyl acetate.

The boroProline esters can also be obtained by treatment of the reaction mixture obtained in the preparation of compound II with anhydrous acid to yield 1-amino-4-bromobutyl boronate pinacol as a salt. Cyclization occurs after neutralizing the salt with base and heating the reaction.

Example 1: Preparation of boroProline-pinacol (H-boroPro-pinacol)

The intermediate, 4-Bromo-1-chlorobutyl boronate pinacol, was prepared by the method in Matteson et al., Organometallics, (3): 1284-1288 (1984), except that conditions were modified for large scale preparations and the pinacol was substitued for the pinanedoil protecting group.

3-bromopropyl boronate pinacol was prepared by hydrogenboronation of allyl bromide (173 ml, 2.00 moles) with catechol borane (240 ml, 2.00 moles). Catechol borane was added to allyl bromide and the reaction heated for 4 hours at 100°C under a nitrogen atmosphere. The product, 3-bromopropyl boronate catechol (bp 95-102°C, 0.25 mm), was isolated in a yield of 49% by distillation. The catechol ester (124 g, 0.52 moles) was transesterified with pinacol (61.5 g, 0.52 moles) by mixing the component in 50 ml of THF and allowing them to stir for 0.5 hours at 0°C and 0.5 hours at room temperature. Solvent was removed by evaporation and 250 ml of hexane added. Catechol was removed as a

crystalline solid. Quantitative removal was achieved by successive dilution to 500 ml and to 1000 ml with hexane and removing crystals at each dilution. Hexane was evaporated and the product distilled to yield 177 g (bp 60 - 64°C, 0.35 mm).

4-Bromo-1-chlorobutyl boronate pinacol was prepared by homologation of the corresponding propyl boronate. Methylene chloride (50.54 ml, 0.713 moles) was dissolved in 500 ml of THF, 1.54 N n-butyllithium in hexane (480 ml, 0.780 moles) was slowly added at -100°C. 3-Bromopropyl boronate pinacol (178 g, 0.713 moles) was dissolved in 500 ml of THG, cooled to the freezing point of the solution, and added to the reaction mixture. Zinc chloride (54.4 g, 0.392 moles) was dissolved in 250 ml of THG, cooled to 0°C, and added to the reaction mixture in several portions. The reaction was allowed to slowly warm to room temperature and to stir overnight. Solvent was evaporated and the residue dissolved in hexane (1 liter) and washed with water (1 liter). Insoluble material was discarded. After drying over anhydrous magnesium sulfate and filtering, solvent was evaporated. The product was distilled to yield 147 g (bp 110 - 112°C, 0.200 mm).

N-Trimethylsilyl-boroProline pinacol was prepared first by dissolving hexamethyldisilizane (20.0 g, 80.0 mmoles) in 30 ml of THF, cooling the solution to -78°c, and adding 1.62 N n-butyllithium in hexane (49.4 ml, 80.0 mmoles). The solution was allowed to slowly warm to room temperature. It was recooled to -78°C and 4-bromo-1-chlorobutyl boronate pinacol (23.9 g, 80.0 mmoles) added in 20 ml of THF. The mixture was allowed to slowly warm to room temperature and to stir overnight. Solvent was removed by evaporation and dry hexane (400 ml) added to yield a precipitant which was removed by filtration under an

nitrogen atmosphere. The filtrate was evaporated and the residue distilled, yielding 19.4 g of the desired product (bp 60 - 62°C, 0.1 - 0.06 mm).

H-boroProline-pinacol.HCl was prepared by cooling N-trimethylsilyl-boroProline-pinacol (16.0 g, 61.7 mmoles) to -78°C and adding 4 N HCL:dioxane 46 ml, 185 mmoles). The mixture was stirred 30 minutes at -78°C and 1 hour at room temperature. Solvent was evaporated and the residue triturated with ether to yield a solid. The crude product was dissolved in chloroform and insoluble material removed by filtration. The solution was evaporated and the product crystallized from ethyl acetate to yield 11.1 g of the desired product (mp 156.5 - 157°C).

Synthesis of boroProline Peptides

General methods of coupling of N-protected peptides and amino acids with suitable side-chain protecting groups to H-boroProline-pinacol are applicable. When needed, side-chain protecting and N-terminal protecting groups can be removed by treatment with anhydrous HCl, HBr, trifluoroacetic acid, or by catalytic hydrogenation. These procedures are known to those skilled in the art of peptide synthesis.

The mixed anhydride procedure of Anderson et al., J. Am. Chem. Soc., 89:5012 (1984) is preferred for peptide coupling. Refering again to Figure 1, the mixed anhydride of an N-protected amino acid or a peptide varying in length from a dipeptide to tetrapeptide is prepared by dissolving the peptide in tetrahydrofuran and adding one equivalent of N-methylmorpholine. The solution is cooled to -20°C and an equivalent of isobutyl chloroformate is added. After 5 minutes, this mixture and one equivalent of triethylamine (or other sterically hindered base) are added to a solution

of H-boroPro-pinacol dissolved in either cold chloroform or tetrahydrofuran.

The reaction mixture is routinely stirred for one hour at -20°C and 1 - 2 hours at room temperature (20°C). Solvent is removed by evaporation, and the residue is dissolved in ethyl acetate. The organic solution is washed with 0.20 N hydrochloric acid, 5% aqueous sodium bicarbonate, and saturated aqueous sodium chloride. The organic phase is dried over anhydrous sodium sulfate, filtered, and evaporated. Products are purified by either silica gel chromatography or gel permeation chromatography using Sephadex™ LH-20 and methanol as a solvent.

Previous studies have shown that the pinacol protecting group can be removed *in situ* by preincubation in phosphate buffer prior to running biological experiments; Kettner et al., J. Biol. Chem. 259: 15106-15114 (1984). Several other methods are also applicable for removing pinacol groups from peptides including boroProline and characterizing the final product. First, the peptide can be treated with diethanolamine to yield the corresponding diethanolamine boronic acid ester, which can be readily hydrolyzed by treatment with aqueous acid or a sulfonic acid substituted polystyrene resin as described in Kettner et al., *id*. Both pinacol and pinanediol protecting groups can be removed by treating with BC13 in methylene chloride as described by Kinder et al., J. Med. Chem., 28: 1917. Finally, the free boronic acid can be converted to the difluoroboron derivative (-BF2) by treatment with aqueous HF as described by Kinder et al., *id*.

Similarly, different ester groups can be introduced by reacting the free boronic acid with various di-hydroxy compounds (for example, those containing heteroatoms such as S or N) in an inert solvent.

Example 2: H-Ala-boroPro

Boc-Ala-boroPro was prepared by mixed anhydride coupling of the N-Boc-protected alanine and H-boroPro prepared as described above. H-Ala-boroPro was prepared by removal of the Boc protecting group at 0°C in 3.5 molar excess of 4 N HCl-dioxane. The coupling and deblocking reactions were performed by standard chemical reaction. Ala-boroPro has a Ki for DP IV of ~1 x 10^{-9}M. Boc-blocked Ala-boroPro has no affinity for DP IV.

The two diastereomers of H-Ala-boroPro-pinacol can be partially separated by silica gel chromatography with 20% methanol in ethyl acetate as eluant. The early fraction appears by NMR analysis to be 95% enriched in one isomer. Because this fraction has more inhibitory power against DP IV than later fractions (at equal concentrations) it is probably enriched in the L-boroPro isomer.

One significant drawback with H-Ala-boroPro as an inhibitor for DP IV is that it decomposes in aqueous solution at neutral pH and room temperature (20 - 25°C) with a half-life of around 0.5 hour. Many dipeptide derivatives with a free N terminal amino group and a functional group (such as a difluoromethyl ketone) on the C-terminus are similarly unstable due to intramolecular reaction. A six member ring is formed between the amino and C-terminal functional groups and undergoes subsequent further reaction, such as hydrolysis. DP IV bound inhibitor is more stable, consistent with the hypothesis that decomposition is due to an intramolecular reaction.

H-Pro-boroPro is more stable than H-Ala-boroPro. The Ki of H-Pro-boroPro for DP IV is about 1 x 10^{-8}M, and it decomposes in aqueous solution at room temperature (20-25°C) with a half life of about 1.5 hours. Although the

affinity of H-Pro-boroPro is about 10-fold less than that of H-Ala-boroPro, the increased stability is advantageous.

Because of the relatively short half life of the above dipeptides inhibitory compounds of the invention are formed as tetrapeptides or longer peptides as shown in the general formula above. These inhibitory compounds are substrates for DP IV yielding the dipeptide inhibitor W-boroPro. These tetrapeptide boronic acids are generally stable and can be administered by any standard procedure to act as a substrate for DP IV and then as a source of a potent DP IV inhibitor. The advantages of such tetrapeptides is that inhibitor is released only in the vicinity of active DP IV. These tetrapeptide boronic acids can be made by the method of mixed anhydride coupling by one of ordinary skill in the art, e.g., Mattason, Organametallics 3:1284 to 1288, 1984.

Test Systems

The following are examples of systems by which the inhibitory activity of the above described inhibitory compounds can be tested on DP IV. As an example H-Ala-boroPro is used to test each of these systems. Inhibitory compounds can be tested by simply substituting them for H-Ala-boroPro.

DP IV is purified from pig kidney cortex by the method of Barth et al., Acta Biol. Med. Germ. (1974) 32:157, and Wolf et al., Acta Biol. Med. Germ. (1978) 37:409, and from human placenta by the method of Puschel et al., E. Eur. J. Biochem. (1982) 126:359. H-Ala-boroPro inhibits both enzymes with a Ki of $\sim 1.0 \times 10^{-9}$M.

Human Peripheral Blood Mononuclear Cells

H-Ala-boroPro was tested for its influence on PHA-induced proliferation of human peripheral blood mononulcear cells. Human peripheral blood mono-nuclear cells were obtained from healthy human donors by Ficoll-Hypaque density gradient centrifugation. The cells are washed three times in RPMI 1640 medium and resuspended to a concentration of a 1×10^6 in RPMI. 10% human serum was used as necessary.

The proliferative response of lymphocytes was measured using 3H-Thymidine incorporation. MNC cells [Ford, W.L. in Handbook of Experimental Immunology edit. by.: D.M. Weir. Blackwell Scientific Publications, Oxford, 1978. p. 23.6] (5×10^3) were distributed into wells of round-bottom microtitre plates (Nunc) and incubated in the presence or absence of various dilutions of antigen, mitogen, lymphokine or other agent of interest. Cells were cultured in a atmosphere of 5% CO_2 in air for 72 hours after which ^{3}H-Thymidine (0.5 uCl/well; 2.0 Ci/mM sp.act., New England Nuclear) was added 6 hours before termination of culture. The cells were harvested with a multiple automatic harvester, and ^{3}H-thymidine incorporation assessed by liquid scintillation counting. ^{3}H thymidine incorporation was determined relative to control values in the absence of inhibitor. Inhibitor was added to give a final concentration of 1×10^{-4}M, but lower concentrations can be used.

HIV gene replication

We examined the effect of H-Ala-boroPro on HIV-1 replication in vitro. The rational for these experiments comes from the reported connection between T-cell activation, IL-2 production, and HIV replication and expression of HIV proteins. For example, inductive signals

associated with HIV replication include mitogens, antigens, lymphokines, and transcriptions factors such as NF-kB, all of which have been shown to be associated with induction of IL-2 production, T-cell activation, or both.

Cell lines used in the present studies include A3.5 cells (a monocyte cell line which is CD4+, HLA-DR+, and CD3-) and peripheral blood mononuclear cells (PBMC). The A3.5 cells grow continuously in culture without exogenous growth factors. PBMC cells require IL-2 for propagation in vitro. Cells were infected with HIV-1IIIB at a multiplicity of infection (moi) of 5×10^{-4} tissue culture infectious dose 50 (TCID50)/cell for both the A3.5 cells and the PMBC cells. Dilutions of inhibitor were made in RPMI-1640 and subsequently passed through a 0.22 um filter. At the start of each experiment, 1×10^6 cells/well, in 24-2311 plates, were infected with HIV-1IIIB at the moi indicated above. Inhibitor was added simultaneously at the appropriate dilutions. All cultures were maintained at 5% CO_2 and 37°C in RPMI-1640 supplemented with penicillin, streptomycin, L-glutamine, hepes buffer, and 20% heat-inactivated fetal calf serum. Cell counts and viability were determined by trypan blue exclusion. Culture supernatants were harvested and assayed for HIV-1 p24 antigen by ELISA (NEN-DuPont, Boston, MA). Fresh media and inhibitor were added on each day. For PBMC cultures, cells were collected from HIV-1 seronegative donors and stimulated with PHA-P (Difco, Detroit, MI; 10 µg/ml) and 10% IL-2 (Electronnucleonics, Silver Spring, MD) 3 days prior to infection with HIV-1. PBMC cultures for all experiments included uninfected and infected cells without inhibitor, uninfected cells with inhibitor at the various concentrations, and infected cells in the presence of 1 um zidovudine (azidothymidine, AZT).

With A3.5 cells H-Ala-boroPro suppresses HIV below detectable levels in a manner similar to the anti-HIV effect of AZT at 1 um. Similar results were observed with the PBMC cells. Thus, inhibitors of this invention have an anti HIV effect. Cell viability assays show that these inhibitors are not cytotoxic even at relatively high concentration (10-3 M for A3.5 cells).

Determination of DP IV Activities in Biological Samples

The ability to determine DP IV activities associated with cells and tissues is highly desirable. For example, it will permit correlations to be made between level of inhibition of DP IV and the magnitude of the observed biological affect, e.g., on cell proliferation, and IL-2 production. Such correlation is helpful in establishing whether or not the biological affect is due to inhibition of DP IV. We have found that such determinations can be reproducibly and reliably made using the readily available chromogenic substrates for DP IV: X-Pro-p-nitroanilides and X-Pro-7-amino-4-trifluoromethyl coumarins (AFC). The AFC substrates are fluorescent and thus provide greater sensitivity. DP IV activity is measured as release of p-nitroanilide spectrophotometrically at 410nM, or using X-Pro-AFC derivatives and measuring fluorescence at 505nM. Reduction in activity in the presence of inhibitor provides an easy test for inhibitory activity.

Use

The inhibitory compounds can be administered in an effective amount either alone or in combination with a pharmaceutically acceptable carrier or diluent.

The above inhibitory compounds are useful for treatment of a wide variety of disease; for example, an autoimmune disease, the pathogenesis of which is dependent

on T cell activity. DP IV plays a role in such autoimmune disease and inhibition of DP IV activity allows regulation of the progress of the disease. Such diseases include arthritis, rejection of transplanted organs, as well as SLE and AIDS. When administered to mammals (e.g., orally, topically, intramuscularly, intraperitoneally, intravenously, parenterally, nasally or by suppository), the inhibitory compounds of this invention enhance the ability of, e.g., the immune system of the mammal, to fight the disease.

Inhibitors of DP IV can suppress IL-2 production and thus diseases in which the production of IL-2 is altered may be treated by use of these inhibitors. These inhibitors can also delay catabolism of growth hormone releasing factor, and block DPIV activity of amoebae and microbial pathogens to allow an immune system to act more efficiently.

The inhibitory compounds or compositions can be administered alone or in combination with one another, or in combination with other therapeutic agents. The dosage level may be between 1 - 500 mg/kg/day.

Other Embodiments

Other embodiments are within the following claims. For example, other inhibitors can be created which mimic the structure of Ala-boroPro. Examples of such inhibitors are shown in Fig. 2 and include Ala-boroPro. These inhibitors generally have a boroPro group, or its equivalent, described above in the Summary of the Invention, and a positively charged amine group. The inhibitors are designed so that minimal interaction of the amine and boroPro groups occurs, and thus no cyclic structure is formed at pH 7.0. These inhibitors interact and/or bind with DPIV, and thereby reduce the DPIV enzymatic activity toward a normal

substrate. These inhibitors are synthesized by procedures well known to those of ordinary skill in this art.

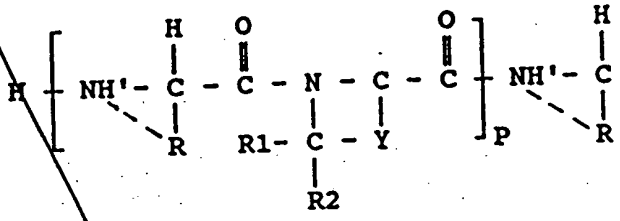
What is claimed is:

- 20 -

1. ~~An inhibitor compound,~~ having the structure

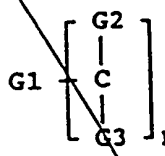
Group I - Group II

where Group I has the structure:



wherein each R, independently, is chosen from the group consisting of the R groups of an amino acid including proline; each broken line, independently, represents a bond to an H or a bond to one said R group, and each H' represents said bond or a hydrogen; p is an integer between 0 and 4 inclusive;

or Group I has the structure:



where n is between 0 and 3 inclusive,

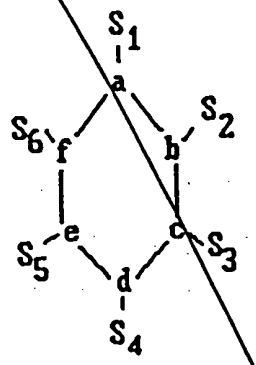
each G2 and G3 independently is H or C1 - 3 alkyl,

G1 is NH_3, $NH-C(=NH_2)$, or

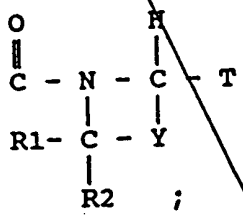
NG_4, where G4 is $C(G_5)(G_6)$

where G5 and G6 can be NH, H, or C1 - 3 alkyl or alkenyl with one or more carbons substituted with a nitrogen; provided that G1 bears a charge and G1 and Group II do not form a covalently bonded ring structure at pH 7.0;

or Group I has the structure:

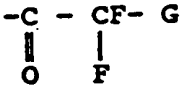


where one or two of said a, b, c, d, e, and f is N and the rest are C, and each S1 - S6 independently is H or C1 - C3 alkyl; where Group II has the structure:

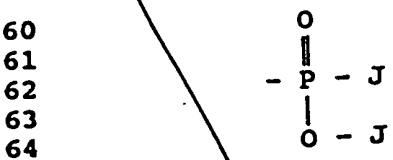


T is a group of the formula:

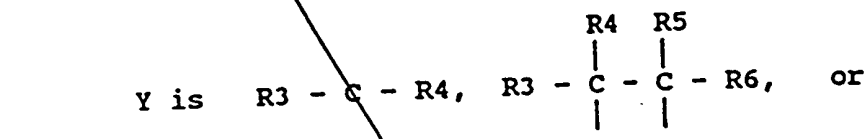
- B- D1, where B is boron and each D1 and D2, independently, is a hydroxyl group or a group which is capable of being hydrolysed to a hydroxyl group in aqueous solution at physiological pH; a group of the formula:

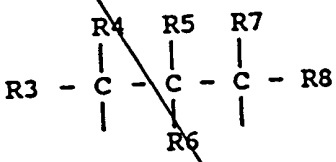


where G is either H, F or an alkyl group containing 1 to 20 carbon atoms and optional heteroatoms which can be N, S, or O; or a phosphonate group of the formula:



where each J, independently, is O-alkyl, N-alkyl, or alkyl, each said O-alkyl, N-alkyl or alkyl comprising 1 - 20 carbon atoms and, optionally, heteroatoms which can be N, S, or O; said T being able to form a complex with the catalytic site of a dipeptidyl-aminopeptidase type IV (DP IV) enzyme;





and each R1, R2, R3, R4, R5, R6, R7, and R8, separately is a group which does not significantly interfere with site specific recognition of said inhibitory compound by said DP IV, and allows said complex to be formed with said DP IV.

2. The compound of claim 1, wherein T is a boronate group.

3. The compound of claim 1, wherein T is a phosphonate group or a trifluoroalkyl ketone group.

4. The compound of claim 1 wherein each R1 - R8 is H.

5. The compound of claim 1 or 2 wherein each R1 and R2 are H, and each Y is CH_2 - CH_2.

6. The compound of claim 5 wherein each R is independently chosen from the R group of proline and alanine.

7. The compound of claim 1, wherein said compound has a binding or dissociation constant to said DP IV of at least 10^{-9}M.

8. The compound of claim 1, wherein said compound has a binding constant to said DP IV of at least 10^{-8}M.

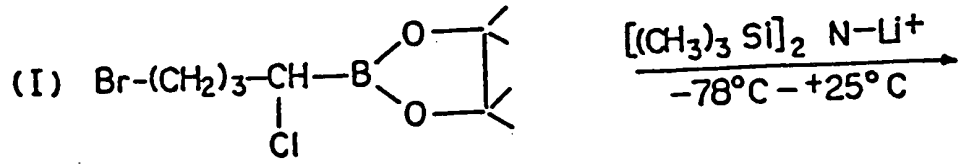
9. The compound of claim 1 admixed within a pharmaceutically acceptable carrier substance.

10. The compound of claim 1 wherein, each D1 and D2 is, independently, F or D1 and D2 together are a ring containing 1 to about 20 carbon atoms, and optionally heteroatoms which can be N, S, or O.

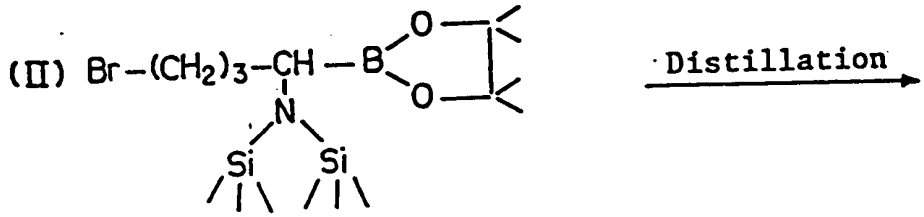
11. A method for inhibiting DP IV in a mammal, comprising administering to said mammal an effective amount of a compound of claim 1.

12. The method of claim 11 wherein said amount is 1 - 500 mg/kg/day.

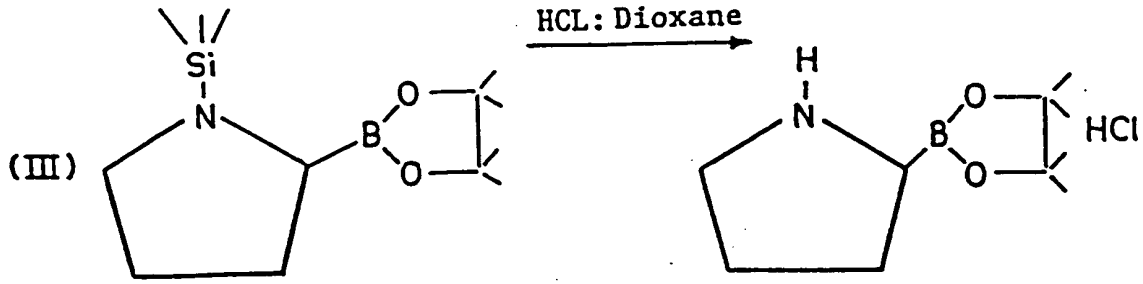
1/2 FIG.1



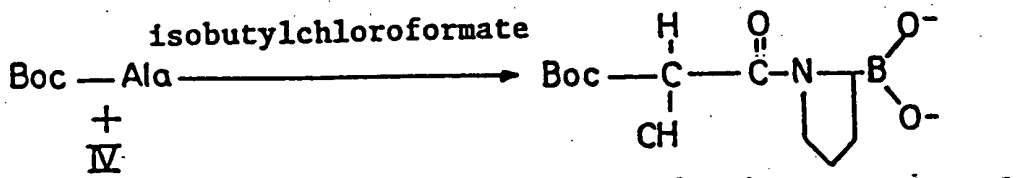
4-bromo-1-chlorobutyl boronate pinacol



4-bromo-1[(bistrimethylsilyl) amino] butyl bornonate pinacol



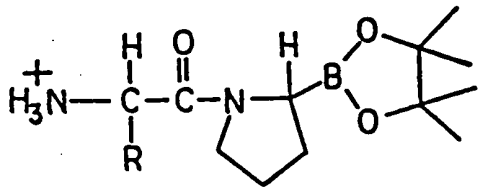
1-trimethylsilyl-boroProline pinacol (IV) boroProline-pinacol-HCL



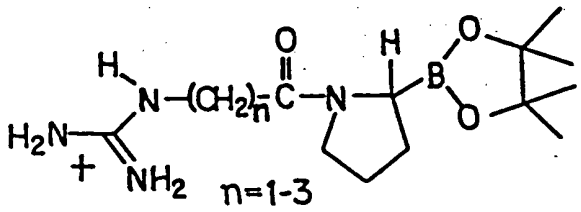
Boc-Ala--boroPro-pinacol

Boc-Ala-boroPro-pinacol —(4N HCl, Dioxane)→ Ala-boroPro-pinacol

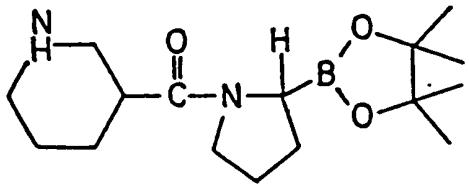
FIG.2

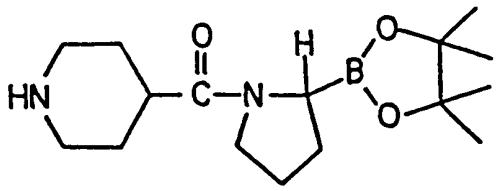


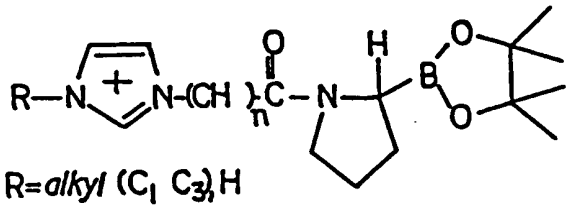
R=H, CH_3



n=1-3

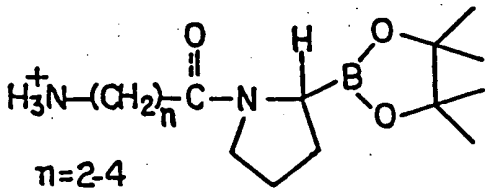






R=*alkyl* (C_1 C_3), H

n=1-3



n=2-4

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): C07K 5/10; A61K 37/02
U.S. CL.: 514/18; 530/330

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
U.S.	514/18; 530/330

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
P, X	US, A 4,935,493 (BACHOVCHIN ET AL.) 19 June 1990, see entire document.	1-12

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
20 May 1991	17 JUL 1991

International Searching Authority	International Examining Officer
ISA/US	Stephen B. Maebius Stephen B. Maebius (vsh)

Form PCT/ISA/210 (second sheet) (rev. 11-87)

PCT INTERNATIONAL APPLICATION TRANSMITTAL LETTER	DATE
	12 April 1991
REGARDING THE INTERNATIONAL APPLICATION OF	DOCKET OR REFERENCE NUMBER
New England Medical Center Hospitals, Inc.	00398/0371

ENTITLED
DIPEPTIDYL-AMINOPEPTIDASE TYPE IV

Certification under 37 CFR 1.10 (if applicable)

FB 461691428
"Express Mail" mailing number

12 April 1991
Date of Deposit

I hereby certify that this application is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10 on the date indicated above and is addressed to the Commissioner of Patents and Trademarks, Washington, D.C. 20231.

Todd Ferrucci
(Typed or printed name of person mailing application)

Todd Ferrucci
(Signature of person mailing application)

To the United States Receiving Office (RO/US):

Accompanying this transmittal letter is the above-identified International application, including a completed Request form (PCT/RO/101). Please process the application according to the provisions of the Patent Cooperation Treaty.

The following requests are made of the RO/US:

1. [X] PREPARATION AND TRANSMITTAL OF CERTIFIED COPY OF PRIORITY DOCUMENTS—Please prepare and transmit to the International Bureau a certified copy of the United States origin priority documents identified in Box VI of the Request form (37 CFR 1.451).

   To cover the cost of copy preparation and certification (37 CFR 1.19(a)(3) and (b)(1)).

   [ ] a (check) (money order) in the amount of $_____ is attached to this transmittal letter.

   [X] the RO/US is hereby authorized to charge the following deposit account no.: 06-1050.

2. [X] CHOICE OF INTERNATIONAL SEARCHING AUTHORITY—It is requested that the International Search be performed by the following International Searching Authority:

   [X] United States Patent and Trademark Office (ISA/US)

   [ ] European Patent Office (ISA/EP)

   The appropriate Search fee for the above-named Authority is indicated on the Fee Calculation Sheet (PCT/RO/101 Annex).

3. [X] SUPPLEMENTAL SEARCH FEES (ONLY WHEN ISA/US CONDUCTS THE INTERNATIONAL SEARCH.)—Please charge any Supplemental Search fees that may be required by the United States International Searching Authority (ISA/US) to deposit account no.: 06-1050.

   I understand that this authorization is subject to any additional information thereto in such instance and that it in no way limits my right to submit a protest against payment of the Supplemental Search fees, but is merely an administrative aid to ensure that the ISA/US has timely complete the Search Report.

NOTE: SUPPLEMENTAL SEARCH FEES FOR ISA/EP ARE PAYABLE DIRECTLY TO THE EUROPEAN PATENT OFFICE

4. [X] DISCLOSURE INFORMATION—In order to assist in screening the accompanying International application for purposes of determining whether a license for foreign transmittal should and could be granted and for other purposes, the following information is supplied:

   A. [ ] There is no prior filed application relating to this invention.

   B. [X] There is a prior application, serial number 510,274 filed on 14 April 1990 which contains subject matter that is

      1. [X] substantially identical to that of the accompanying International application.

      2. [ ] less than that of the accompanying International application. The additional subject matter of the International application appears on pages(s) and line(s) _____.

      3. [ ] more than that of the accompanying International application.

   C. [ ] Disclosure information cannot be covered by the language of Points 4A or 4B above due to the involvement of several prior applications or for other reasons. A separate sheet on which the disclosure information is explained is attached to this transmittal letter.

5. [X] REQUEST FOR FOREIGN TRANSMITTAL LICENSE—According to the provisions of 35 U.S.C. 184 and 37 CFR 5.11, a license to transmit the accompanying International application to foreign agencies or international authorities is hereby requested

INTERNATIONAL APPLICATION UNDER THE PATENT COOPERATION TREATY

REQUEST

THE UNDERSIGNED REQUESTS THAT THE PRESENT INTERNATIONAL APPLICATION BE PROCESSED ACCORDING TO THE PATENT COOPERATION TREATY

(The following is to be filled in by the receiving Office)

INTERNATIONAL APPLICATION No:

INTERNATIONAL FILING DATE:

(Stamp) Name of receiving Office and "PCT International Application"

Applicant's or Agent's File Reference (indicated by applicant if desired) 00398/0371

Box No. I TITLE OF INVENTION

DIPEPTIDYL-AMINOPEPTIDASE TYPE IV

Box No. II APPLICANT (WHETHER OR NOT ALSO INVENTOR); DESIGNATED STATES FOR WHICH HE/SHE/IT IS APPLICANT. Use this box for indicating the applicant or, if there are several applicants, one of them. If more than one person (includes, where applicable, a legal entity) is involved, continue in Box No. III.

The person identified in this box is (check one only): [ ] applicant and inventor* [X] applicant only

Name *and* address:**

NEW ENGLAND MEDICAL CENTER HOSPITALS, INC.
750 Washington Street
Boston, Massachusetts 02111
United States of America

Telephone number: (including area code)

Telegraphic address:

Teleprinter address:

Country of nationality: US

Country of residence:*** US

The person identified in this box is *applicant* for the purposes of (check one only):

[X] all designated States [ ] all designated States except the United States of America [ ] the United States of America only [ ] the States indicated in the "Supplemental Box"

Box No. III FURTHER APPLICANTS, IF ANY; (FURTHER) INVENTORS, IF ANY; DESIGNATED STATES FOR WHICH THEY ARE APPLICANTS (IF APPLICABLE). A separate sub-box has to be filled in in respect of each person (includes, where applicable, a legal entity). If the following two sub-boxes are insufficient, continue in the "Supplemental Box," (giving there for each additional person the same indications as those requested in the following two sub-boxes) or by using a "continuation sheet."

The person identified in this sub-box is (check one only): [ ] applicant and inventor* [X] applicant only [ ] inventor only*

Name *and* address:**

TUFTS UNIVERSITY SCHOOL OF MEDICINE
136 Harrison Avenue
Boston, Massachusetts 02111
United States of America

If the person identified in this sub-box is *applicant (or applicant and inventor)*, indicate also:

Country of nationality: US

Country of residence:*** US

and whether that person is *applicant* for the purposes of (check one only):

[X] all designated States [ ] all designated States except the United States of America [ ] the United States of America only [ ] the States indicated in the "Supplemental Box"

The person identified in this sub-box is (check one only): [ ] applicant and inventor* [ ] applicant only [X] inventor only*

Name *and* address:**

BACHOVCHIN, William W.
71 Warwick Road
Melrose, Massachusetts 02176
United States of America

If the person identified in this sub-box is *applicant (or applicant and inventor)*, indicate also:

Country of nationality:

Country of residence:***

and whether that person is *applicant* for the purposes of (check one only):

[ ] all designated States [ ] all designated States except the United States of America [ ] the United States of America only [ ] the States indicated in the "Supplemental Box"

* If the person indicated as "applicant and inventor" or as "inventor only" is not an *inventor* for the purposes of all the designated States, give the necessary indications in the "Supplemental box."

** Indicate the name of a natural person by giving his/her family name first followed by the given name(s). Indicate the name of a legal entity by [illegible] include both the postal code (if any) and the country (name).

Box No. III CONTINUATION (IF REQUIRED) FURTHER APPLICANTS, IF ANY; (FURTHER) INVENTORS, IF ANY; DESIGNATED STATES FOR WHICH THEY ARE APPLICANTS (IF APPLICABLE). A separate sub-box has to be filled in in respect of each person (includes, where applicable, a legal entity).

The person identified in this sub-box is (check one only): ☐ applicant and inventor* ☐ applicant only ☒ inventor only*

Name *and* address:**

PLAUT, Andrew G.
c/o New England Medical Center
750 Washington Street
Boston, Massachusetts 02111
United States of America

If the person identified in this sub-box is *applicant (or applicant and inventor)*, indicate also:

Country of nationality: Country of residence:***

and whether that person is *applicant* for the purposes of (check one only):

☐ all designated States ☐ all designated States except the United States of America ☐ the United States of America only ☐ the States indicated in the "Supplemental Box"

The person identified in this sub-box is (check one only): ☐ applicant and inventor* ☐ applicant only ☒ inventor only*

Name *and* address:**

FLENTKE, George R.
c/o Tufts University School of Medicine
136 Harrison Avenue
Boston, Massachusetts 02111
United States of America

If the person identified in this sub-box is *applicant (or applicant and inventor)*, indicate also:

Country of nationality: Country of residence:***

and whether that person is *applicant* for the purposes of (check one only):

☐ all designated States ☐ all designated States except the United States of America ☐ the United States of America only ☐ the States indicated in the "Supplemental Box"

The person identified in this sub-box is (check one only): ☐ applicant and inventor* ☐ applicant only ☐ inventor only*

Name *and* address:**

If the person identified in this sub-box is *applicant (or applicant and inventor)*, indicate also:

Country of nationality: Country of residence:***

and whether that person is *applicant* for the purposes of (check one only):

☐ all designated States ☐ all designated States except the United States of America ☐ the United States of America only ☐ the States indicated in the "Supplemental Box"

The person identified in this sub-box is (check one only): ☐ applicant and inventor* ☐ applicant only ☐ inventor only*

Name *and* address:**

If the person identified in this sub-box is *applicant (or applicant and inventor)*, indicate also:

Country of nationality: Country of residence:***

and whether that person is *applicant* for the purposes of (check one only):

☐ all designated States ☐ all designated States except the United States of America ☐ the United States of America only ☐ the States indicated in the "Supplemental Box"

* If the person indicated as "applicant and inventor" or as "inventor only" is not an *inventor* for the purposes of all the designated States, give the necessary indications in the "Supplemental box."

** Indicate the name of a natural person by giving his/her family name first followed by the given name(s). Indicate the name of a legal entity by its full official designation. In the address, include both the postal code (if any) and the country (name).

*** If residence is not indicated, it will be assumed that the country of residence is the same as the country indicated in the address.

Box No. IV AGENT (IF ANY) OR COMMON REPRESENTATIVE (IF ANY); ADDRESS FOR NOTIFICATION (IN CERTAIN CASES). A common representative may be appointed only if there are several applicants and if no agent is or has been appointed; the common representative must be one of the applicants.
The following person (includes, where applicable, a legal entity) is hereby/has been appointed as agent or common representative to act on behalf of the applicant(s) before the competent International Authorities:

Name *and* address, including postal code and country:

If the space below is used instead for an address for notifications, mark here ☐

CLARK, Paul T.
Fish & Richardson
225 Franklin Street
Boston, Massachusetts 02110
United States of America

Telephone number: (including area code) 617-542-5070
Telegraphic address: FishRich Boston
Teleprinter address: 617-542-8906

Box No. V DESIGNATION OF GROUPS OF STATES OR STATES (1); CHOICE OF CERTAIN KINDS OF PROTECTION OR TREATMENT. The following designations are hereby made (please mark the applicable check-boxes):

Regional Patent

[X] EP European Patent(2): AT Austria, BE Belgium, CH and LI Switzerland and Liechtenstein, DE Germany (Federal Republic of), DK Denmark, ES Spain, FR France, GB United Kingdom, IT Italy, LU Luxembourg, NL Netherlands, SE Sweden,
and any other State which is a Contracting State of the European Patent Convention and of the PCT

☐ OA OAPI Patent: Benin, Burkina Faso, Cameroon, Central African Republic, Chad, Congo, Gabon, Mali, Mauritania, Senegal, Togo,
and any other State which is a Contracting State of OAPI and of the PCT; if other OAPI title desired, specify on dotted line(3): .....

National Patent (if other kind of protection or treatment desired, specify on dotted line(3))

☐ AT Austria(3) .....
☐ AU Australia(3) .....
☐ BB Barbados
☐ BG Bulgaria(3) .....
☐ BR Brazil(3) .....
[X] CA Canada
☐ CH and LI Switzerland and Liechtenstein
☐ DE Germany (Federal Republic of)(3) .....
.....
☐ DK Denmark
☐ ES Spain(3) .....
☐ FI Finland
☐ GB United Kingdom
☐ HU Hungary
[X] JP Japan(3) .....
☐ KP Democratic People's Republic of Korea(3) .....
.....
☐ KR Republic of Korea(3) .....
☐ LK Sri Lanka
☐ LU Luxembourg(3) .....
☐ MC Monaco(3) .....
☐ MG Madagascar
☐ MW Malawi(3) .....
☐ NL Netherlands
☐ NO Norway
☐ RO Romania
☐ SD Sudan
☐ SE Sweden
☐ SU Soviet Union(3) .....
.....
☐ US United States of America(3) .....
.....

Space reserved for designating States (for the purposes of a national patent) which have become party to the PCT after the issuance of this sheet:

.....

(1) The applicant's choice of the order of designations may be indicated by marking the check-boxes with sequential arabic numerals (see also the "Notes to Box No. V").
(2) The selection of particular States for a European patent can be made upon entering the national (regional) phase before the European Patent Office (see also the "Notes to Box No. V").
(3) If another kind of protection or a title of addition or, in the United States of America, treatment as a continuation or a continuation-in-part is desired, specify according to the instructions given in the "Notes to Box No. V."

See notes on accompanying sheet

Box No. VI PRIORITY CLAIM (IF ANY). The priority of the following earlier application(s) is hereby claimed:

Country (country in which it was filed if national application; one of the countries for which it was filed if regional or international application)	Filing Date (day, month, year)	Application No.	Office of Filing (fill in only if the earlier application is an international application or a regional application)
(1) US	(14.04.90) 14 April 1990	510,274	
(2)			
(3)			

(Letter codes may be used to indicate country and/or Office of filing)

When the earlier application was filed with the Office which, for the purposes of the present international application, is the receiving Office, the applicant may, *against payment of the required fee*, ask the following:

[X] the receiving Office is hereby requested to prepare and transmit to the International Bureau a certified copy of the above-mentioned earlier application/of the earlier applications identified above by the numbers (insert the applicable numbers) .....

Box No. VII EARLIER SEARCH (IF ANY). Fill in where a search (international, international-type or other) by the International Searching Authority has already been requested (or completed) and the said Authority is now requested to base the international search, to the extent possible, on the results of the said earlier search. Identify such search or request either by reference to the relevant application (or the translation thereof) or by reference to the search request.

International application number or number and country (or regional Office) of other application:

International/regional/national filing date

Date of request for search:

Number (if available) given to search request:

Box No. VIII SIGNATURE OF APPLICANT(S) OR AGENT

NEW ENGLAND MEDICAL CENTER HOSPITLAS, INC.
TUFTS UNIVERSITY SCHOOL OF MEDICINE

Reg No. 30,135

Paul T. Clark
Attorney for Applicant

If the present Request form is signed on behalf of any applicant by an agent, a separate power of attorney appointing the agent and signed by the applicant is required. If in such case it is desired to make use of a general power of attorney (deposited with the receiving Office), a copy thereof must be attached to this form.

Box No.IX CHECK LIST (To be filled in by the Applicant)

This international application contains the following number of sheets:

1. request	4	sheets
2. description	19	sheets
3. claims	4	sheets
4. abstract	1	sheets
5. drawings	2	sheets
Total	30	sheets

Figure number ..... of the drawings (if any) is suggested to accompany the abstract for publication.

This international application as filed is accompanied by the items checked below:

1. [ ] separate signed power of attorney
2. [ ] copy of general power of attorney
3. [ ] priority document(s) (see Box No. VI)
4. [ ] receipt of the fees paid or revenue stamps
5. [X] cheque for the payment of fees
6. [ ] request to charge deposit account
7. [X] other document (specify) Transmittal Letter Fee Calculation sheet

(The following is to be filled in by the receiving Office)

1. Date of actual receipt of the purported international application:
2. Corrected date of actual receipt due to later but timely received papers or drawings completing the purported international application:
3. Date of timely receipt of the required corrections under Article 11 of the PCT:
4. Drawings [ ] Received [ ] No Drawings

(The following is to be filled in by the International Bureau)

Date of receipt of the record copy:

THIS SHEET IS NOT PART OF AND DOES NOT COUNT AS A SHEET OF THE INTERNATIONAL APPLICATION

APPLICANT
NEW ENGLAND MEDICAL CENTER HOSPITLAS, INC., et al.

INTERNATIONAL APPLICATION NUMBER (to be filled in by the receiving Office)

DATE STAMP OF RECEIVING OFFICE

This column for use by receiving Office

FEE CALCULATION SHEET[1]

FEES SUBMITTED OR TO BE CHARGED TO DEPOSIT ACCOUNT

I. TRANSMITTAL FEE[2] ..... 170 T

380 S

II. SEARCH FEE[3] .....

International search to be effected by .....
(Please indicate, but only if the applicant has the choice between two or more International Searching Authorities, the name of the Authority to which the international application is to be transmitted. Note that the amount of the search fee depends on the identity of the International Searching Authority.)

III. INTERNATIONAL FEE[4]

BASIC FEE[5]

Indicate the number of SHEETS contained in the international application 30.

first 30 sheets ..... 559 b_1

remaining _____ sheets x _____ = _____ b_2

Add amounts entered in boxes b_1 and b_2 and enter total in box B. This figure is the amount of the BASIC FEE ..... 559 B

DESIGNATION FEES[5]

Indicate the number of NATIONAL PATENTS which have been sought and multiply by the amount of the designation fee. 2 x 135 = 270 d_1

Indicate the number of REGIONAL PATENTS which have been sought and multiply by the amount of the designation fee. 1 x 135 = 135 d_2

Add amounts entered in boxes d_1 and d_2 and enter total in box D (if that total exceeds the figure which corresponds to the amount of the designation fee multiplied by ten, enter the latter figure in Box D)[6]. This figure is the amount of the DESIGNATION FEES ..... 405 D

Add amounts entered in boxes B and D, and enter total in box I. This figure is the amount of the INTERNATIONAL FEE ..... 964 I

IV. TOTAL OF PRESCRIBED FEES SUBMITTED OR TO BE CHARGED TO DEPOSIT ACCOUNT

Add amounts entered in boxes T, S and I, and enter total in the TOTAL box. This figure is the total amount of the PRESCRIBED FEES SUBMITTED OR TO BE CHARGED TO DEPOSIT ACCOUNT ..... 1514 TOTAL

THE APPLICANT MAY PAY THE PRESCRIBED FEES BY [CHEQUE, POSTAL MONEY ORDER, BANK DRAFT, CASH, REVENUE STAMPS, COUPONS, ETC.]. PAYMENT SHOULD BE MADE IN THE PRESCRIBED CURRENCY TO THE [ACCOUNT OF, ACCOUNT INDICATED BELOW OF, ORDER OF] THE RECEIVING OFFICE. PAYMENT MAY ALSO BE MADE BY AUTHORIZATION TO CHARGE A DEPOSIT ACCOUNT AT THE RECEIVING OFFICE IF THE LATTER HAS A DEPOSIT ACCOUNT SYSTEM.

DEPOSIT ACCOUNT AUTHORIZATION[7]

☐ The RO/ is hereby authorized to charge the total fees indicated above to my deposit account.

☒ The RO/ US is hereby authorized to charge any deficiency or credit any overpayment in the total fees indicated above to my deposit account.

☒ The RO/ US is hereby authorized to charge the fee for preparation and transmittal of the priority document to the International Bureau of WIPO to my deposit account.

06-1050
Deposit Account Number

12 Dec
Date

Reg No 39,175
Signature Paul T. Clark

CHAPTER II

PATENT COOPERATION TREATY
DEMAND
UNDER ARTICLE 31 OF THE PATENT COOPERATION TREATY:

THE UNDERSIGNED REQUESTS THAT THE INTERNATIONAL APPLICATION SPECIFIED BELOW BE THE SUBJECT OF INTERNATIONAL PRELIMINARY EXAMINATION ACCORDING TO THE PATENT COOPERATION TREATY

Box No. I IDENTIFICATION OF THE INTERNATIONAL APPLICATION

Applicant's or Agent's File Reference (indicated by applicant if desired): 00398/0371

International Application No.	International Filing Date	(Earliest) Priority Date
PCT/US91/02519	12 April 1991	14 April 1990

Title of Invention

DIPEPTIDYL-AMINOPEPTIDASE TYPE IV

Box No. II APPLICANT(S). Further applicants are indicated on a continuation sheet ☐

Name *and* address, including postal code and country:

New England Medical Center Hospitals, Inc.
750 Washington Street
Boston, Massachusetts 02111
United States of America

State of nationality:

State of residence:*

Telephone number (including area code):

Telegraphic address:

Teleprinter address:

Name *and* address, including postal code and country:

Tufts University School of Medicine
136 Harrison Avenue
Boston, Massachusetts 02111
United States of America

State of nationality:

State of residence:*

Box No. III AGENT OR COMMON REPRESENTATIVE (IF ANY); ADDRESS FOR NOTIFICATIONS (IN CERTAIN CASES)

The following named agent or common representative

1. [X] has been appointed earlier and represents the applicant also for international preliminary examination
2. [ ] is hereby appointed and any earlier appointment of an agent is hereby revoked
3. [ ] is hereby appointed, in addition to the agent(s) appointed earlier, for the procedure before the International Preliminary Examining Authority

If the space below is used instead for an address for notifications, mark here. ☐

Name *and* address, including postal code and country:

CLARK, Paul T.
Fish & Richardson
225 Franklin Street
Boston, Massachusetts 02110-2804
United States of America

Telephone number (including area code): (617)542-5070

Telegraphic address: FishRich Boston

Teleprinter address: (617)542-8906

* If residence is not indicated, it will be assumed that the State of residence is the same as the State indicated in the address.

Box No. IV DECLARATION CONCERNING AMENDMENTS OF THE CLAIMS

Applicant wishes international preliminary examination to start promptly on the basis of the claims

- [X] as filed (amendments under Article 19 have not been made and will not be made)
- [ ] as amended under Article 19
- [ ] as specified on the attached sheet

Box No. V ELECTION OF STATES

The following designated States are hereby elected (please mark the applicable check-boxes):

Regional Patent

- [X] EP European Patent: AT Austria, BE Belgium, DE Germany, DK Denmark, FR France, GB United Kingdom, IT Italy, LU Luxembourg, NL Netherlands, SE Sweden, and any other State which is a Contracting State of the European Patent Convention and of the PCT (including Chapter II thereof).
- [ ] OA OAPI Patent: Benin, Burkina Faso, Cameroon, Central African Republic, Chad, Congo, Gabon, Mali, Mauritania, Senegal, Togo, and any other State which is a Contracting State of the OAPI and of the PCT (including Chapter II thereof).

National Patent

- [ ] AT Austria
- [ ] AU Australia
- [ ] BB Barbados
- [ ] BG Bulgaria
- [ ] BR Brazil
- [X] CA Canada
- [ ] DE Germany
- [ ] DK Denmark
- [ ] FI Finland
- [ ] GB United Kingdom
- [ ] HU Hungary
- [X] JP Japan
- [ ] KP Democratic People's Republic of Korea
- [ ] KR Republic of Korea
- [ ] LK Sri Lanka
- [ ] LU Luxembourg
- [ ] MC Monaco
- [ ] MG Madagascar
- [ ] MW Malawi
- [ ] NL Netherlands
- [ ] NO Norway
- [ ] PL Poland
- [ ] RO Romania
- [ ] SD Sudan
- [ ] SE Sweden
- [ ] SU Soviet Union
- [ ] US United States of America

Space reserved for electing States which have become party to the PCT (including Chapter II thereof) or bound by Chapter II of the PCT after the issuance of this sheet:

....

"Express Mail" mailing label number EB68711
Date of Deposit November 15, 1991
I hereby ...
Washington, D.C. 20231
Maria Stefano
Maria Stefano

Box No. VI SIGNATURE

New England Medical Center Hospitals, Inc.
Tufts University School of Medicine

Reg. No. 39123
Paul T. Clark
Attorney for Applicants

(The following is to be filled in by the International Preliminary Examining Authority)

1. Date of actual receipt of DEMAND:
2. Adjusted date of receipt of DEMAND due to CORRECTIONS under Rule 60.1(b):

Form PCT/IPEA/401 (last sheet) (January 1991)

See notes on accompanying sheet

FEE CALCULATION SHEET
ANNEX TO THE DEMAND FOR INTERNATIONAL PRELIMINARY EXAMINATION

APPLICANT	For use by IPEA
New England Medical Center Hospitals, Inc. et al.	

INTERNATIONAL APPLICATION No.	DATE STAMP OF THE IPEA
PCT/US91/02519	

I. PRELIMINARY EXAMINATION FEE	400	P	
II. HANDLING FEE	150	H	
III. TOTAL OF PRESCRIBED FEES SUBMITTED OR TO BE CHARGED TO DEPOSIT ACCOUNT Add the amounts entered in Boxes P and H, and enter the total in the TOTAL Box. THIS FIGURE IS THE TOTAL AMOUNT OF THE PRESCRIBED FEES SUBMITTED OR TO BE CHARGED TO THE DEPOSIT ACCOUNT.....	550 TOTAL		

THE APPLICANT MAY PAY THE PRESCRIBED FEES BY ICHEQUE, POSTAL MONEY ORDER, BANK DRAFT, CASH, REVENUE STAMPS, COUPONS, ETC.I. PAYMENT SHOULD BE MADE IN THE PRESCRIBED CURRENCY TO THE IACCOUNT OF, ACCOUNT INDICATED BELOW OF, ORDER OFI THE INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY. PAYMENT MAY ALSO BE MADE BY AUTHORIZATION TO CHARGE A DEPOSIT ACCOUNT AT THE INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY IF THE LATTER HAS A DEPOSIT ACCOUNT SYSTEM.

DEPOSIT ACCOUNT AUTHORIZATION

— The IPEA/ is hereby authorized to charge the total fees indicated above to my deposit account.

X The IPEA/US is hereby authorized to charge any deficiency or credit any overpayment in the total fees indicated above to my deposit account.

06-1050
Deposit Account Number

November 13, 1991
Date

Signature Paul T. Clark Reg No 39125

See notes on reverse side

Form PCT/IPEA/401 (Annex) (January 1991)

PATENT COOPERATION TREATY

UNITED STATES INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY (IPEA/US)

Paul T. Clark
Fish and Richardson
225 Franklin Street
Boston, Massachusettes 02110-2804

WRITTEN OPINION

issued pursuant to PCT Rules 66.2[1] or 66.4 (a)[2]

Inscribe NAME and ADDRESS of the AGENT and if there is no agent, of the APPLICANT

DATE OF MAILING by the International Preliminary Examining Authority: 13 APR 1992

APPLICANT'S OR AGENT'S FILE REFERENCE: 003981/0371

IDENTIFICATION OF THE INTERNATIONAL APPLICATION

International Application No.	International Filing Date
PCT/US91/02519	12 April 1991

Applicant (Name): NEW ENGLAND MEDICAL CENTER HOSPITALS, INC.

Receiving Office	Priority Date Claimed
RO/US	14 April 1990

DF4

Docketed for June 13

WRITTEN OPINION

With reference to the above-identified international application, this constitutes the first (first, etc.) written opinion by this International Preliminary Examining Authority.

I. BASIS OF OPINION

The examination is being carried out on the following application documents:

- ☐ the application documents as filed
- ☒ description, pages 1-19, as originally filed
- ☐ description, pages _____, filed with your letter of _____
- ☒ claim(s) 1-12, as originally filed
- ☐ claim(s) _____, filed with your letter of _____
- ☒ drawings, sheet/fig. _____, as originally filed
- ☒ drawings, sheet/fig. 2/2, filed with your letter of 23 May 1991
- ☐ This opinion has been established as if the amendments indicated on the extra sheet have not been made, since, for the reasons indicated, they have been considered to go beyond the disclosure as filed.

II. NON ESTABLISHMENT OF OPINION ON NOVELTY, INVENTIVE STEP AND INDUSTRIAL APPLICABILITY

The question whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), and to be industrially applicable will not for the reasons indicated below be gone into in respect of:

1. ☐ The above-identified international application.
2. ☐ claims Nos. _____ (specify particular claims).

☐ Said international application, or said claims Nos. _____ relate to the following subject matter[3] which does not require an international preliminary examination. (specify)

Docketed
Action Code PCTOP
Base Date 04-13-92
Due Date _____
Final Deadline 06-13-92
Docketed by wtf

☐ The description, claims, or drawings (indicate particular elements) or said claims Nos. _____ are so unclear that no meaningful opinion could be formed.[3]

☐ The claims, or said claims Nos. _____ are so inadequately supported by the description that no meaningful opinion could be formed.[3]

WRITTEN OPINION (continued)

III. NEGATIVE STATEMENT IN REGARD TO NOVELTY, INVENTIVE STEP AND INDUSTRIAL APPLICABILITY OF CLAIMS

The statement under Article 35 (2) should be negative in respect of the claims indicated below. The criteria not satisified in respect of such claims are indicated by the letter abbreviation: N (for Novelty); IS (for Inventive Step); and IA (for Industrial Applicability).

IV. CITATIONS AND EXPLANATIONS IN REGARD TO NOVELTY, INVENTIVE STEP AND INDUSTRIAL APPLICABILITY OF CLAIMS

No. of Claim / Relevent Supporting Documents Cited / Explanation

Claims 1-12 meet the criteria of PCT Article 33(2)-(4) since the claimed inhibitor compounds and method for inhibiting DP IV in a mammal is neither taught by nor fairly suggested by the prior art.

See notes on reverse side

WRITTEN OPINION (continued)

V. CERTAIN DEFECTS IN THE INTERNATIONAL APPLICATION

The following defects in the form or contents of the above-identified international application under the Treaty or the Regulations have been noted.

VI. CERTAIN OBSERVATIONS ON THE INTERNATIONAL APPLICATION

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are notified:

Claim 1 is indefinite under 35 USC 112 second paragraph for lack of clarity. The claim does not specifically define each of R1, R2, R3, R4, R5, R6, R7, R8 and R9. The definition "with at least 3 [illegible] of an amino acid [illegible] position" is indefinite since what groups of the amino acids are 3 groups. Also the term "[illegible] position" should be deleted since it does not further limit the term "amino acids."

VII. INVITATION

APPLICANT IS INVITED TO SUBMIT A WRITTEN REPLY ACCOMPANIED, WHERE APPROPRIATE, BY AMENDMENTS[4] WITHIN two MONTHS/ -- DAYS OF THE DATE OF MAILING INDICATED ON THE FIRST SHEET.

Any inquiry concerning this communication should be directed to examiner Lester L. Lee at telephone number 703-308-3994.

The time limit set for response to a Written Opinion may not be extended. 37 CFR 1.484(d). Any response received after the expiration of the time limit set in the Written Opinion will not be considered in preparing the International Preliminary Examination Report.

THE INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

Address Only:
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. ATTN: IPEA/US

Authorized Officer
Lester L. Lee
Lester L. Lee

NOTES TO FORM PCT/IPEA/408

These Notes are intended to facilitate the use of the present form. For full information, see the text of the Patent Cooperation Treaty and the texts of the Regulations and the Administrative Instructions under that Treaty. In case of discrepancy between these Notes and the said texts, the latter are applicable. "Article" refers to Articles of the Treaty, "Rule" refers to Rules of the Regulations and "Section" refers to Sections of the Administrative Instructions.

(1) "If the International Preliminary Examining Authority

(i) considers that the international application has any of the defects described in Article 34(4),

(ii) considers that the international preliminary examination report should be negative in respect of any of the claims because the invention claimed therein does not appear to be novel, does not appear to involve an inventive step (does not appear to be non-obvious), or does not appear to be industrially applicable,

(iii) notices that there is some defect in the form or contents of the international application under the Treaty or these Regulations,

(iv) considers that any amendment goes beyond the disclosure in the international application as filed, or

(v) wishes to accompany the international preliminary examination report by observations on the clarity of the claims, the description, and the drawings, or the question whether the claims are fully supported by the description,

the said Authority shall notify the applicant accordingly in writing. Where the national law of the national Office acting as International Preliminary Examining Authority does not allow multiple dependent claims to be drafted in a manner different from that provided for in the second and third sentences of Rule 6.4(a), the International Preliminary Examining Authority may, in case of failure to use that manner of claiming, apply Article 34(4)(b). In such case, it shall notify the applicant accordingly in writing." *(Rule 66.2(a))*

"The notification shall fully state the reasons for the opinion of the International Preliminary Examining Authority." *(Rule 66.2(b))*

"The notification shall invite the applicant to submit a written reply together, where appropriate, with amendments." *(Rule 66.2(c))*

"The notification shall fix a time limit for the reply. The time limit shall be reasonable under the circumstances. It shall normally be 2 months after the date of notification. In no case shall it be shorter than 1 month after the said date. It shall be at least 2 months after the said date where the international search report is transmitted at the same time as the notification. In no case shall it be more than 3 months after the said date." *(Rule 66.2(d))*

(2) "If the International Preliminary Examining Authority wishes to issue one or more additional written opinions, it may do so, and Rules 66.2 and 66.3 shall apply." *(Rule 66.4(a))*

(3) "If the International Preliminary Examining Authority considers

(i) that the international application relates to a subject matter on which the International Preliminary Examining Authority is not required, under the Regulations, to carry out an international preliminary examination, and in the particular case decides not to carry out such examination, or

(ii) that the description, the claims, or the drawings, are so unclear, or that the claims are so inadequately supported by the description, that no meaningful opinion can be formed on the novelty, inventive step (non-obviousness), or industrial applicability, of the claimed invention,

the said Authority shall not go into the questions referred to in Article 33 (1) and shall inform the applicant of this opinion and the reasons therefor." *(Article 34 (4)(a))*

Rule 67 entitled "Subject Matter Under Article 34 (4)(a)(i)" read as follows:

"No International Preliminary Examining Authority shall be required to carry out an international preliminary examination on an international application if, and to the extent to which, its subject matter is any of the following:

(i) scientific and mathematical theories,

(ii) plant or animal varieties or essentially biological processes for the production of plants and animals, other than microbiological processes and the products of such processes,

(iii) schemes, rules or methods of doing business, performing purely mental acts or playing games,

(iv) methods for treatment of the human or animal body by surgery or therapy, as well as diagnostic methods,

(v) mere presentations of information,

(vi) computer programs to the extent that the International Preliminary Examining Authority is not equipped to carry out an international preliminary examination concerning such programs."

(4) "The applicant may respond to the invitation referred to in *Rule 66.2(c)* of the International Preliminary Examining Authority by making amendments or, if he disagrees with the opinion of that Authority, by submitting arguments, as the case may be, or do both." *(Rule 66.3(a))*

"Any response shall be submitted directly to the International Preliminary Examining Authority." *(Rule 66.3(b))*

"On the request of the applicant, the International Preliminary Examining Authority may give him one or more additional opportunities to submit amendments or arguments." *(Rule 66.4 (b))*

"The applicant shall be required to submit a replacement sheet for every sheet of the international application which, on account of an amendment, differs from the sheet originally filed. The letter accompanying the replacement sheets shall draw attention to the differences between the replaced sheets and the replacement sheets. To the extent that any amendment results in the cancellation of an entire sheet, that amendment shall be communicated in a letter." *(Rule 66.8(a))*

"If the international application has been filed in a language other than the language in which it is published, any amendment, as well as any letter referred to in Rule 66.8 (a), shall be submitted in the language of publication." *(Rule 66.9)*

"Amendments to the claim under Article 19 or Article 34 (2) may be made either by canceling one or more entire claims, by adding one or more new claims or by amending the text of one or more of the claims as filed. All the claims appearing on a replacement sheet shall be numbered in arabic numerals. Where a claim is canceled, no renumbering of the other claims shall be required. In all cases where claims are renumbered, they shall be renumbered consecutively." *(Section 205 (a))*

"The applicant shall, in the letter referred to in the second and third sentences of Rule 46.5 (a) or of Rule 66.8 (a), indicate the differences between the claims as filed and the claims as amended. He shall, in particular, indicate in the said letter, in connection with each claim appearing in the international application (it being understood that identical indications concerning several claims may be grouped), whether:

(i) the claim is unchanged;

(ii) the claim is cancelled;

(iii) the claim is new;

(iv) the claim replaces one or more claims as filed;

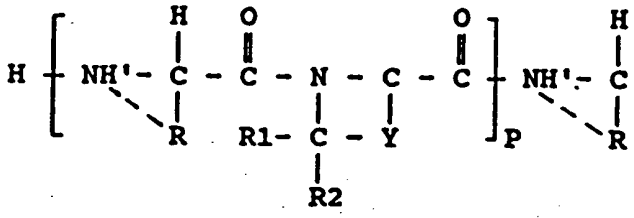
(v) the claim is the result of the division of a claim as filed." *(Section 205 (b))*

The attention of the applicant is also drawn to the examples given, in respect of the amendments of claims, in the Notes to Form PCT/ISA/220, which he received from the International Searching Authority; these examples are also valid in respect of amendments made in the course of the international preliminary examination.

1. Use of compound having the structure

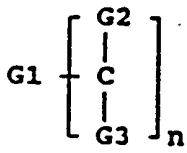
Group I - Group II

where Group I has the structure:



wherein each R, independently, is chosen from the group consisting of the R groups of an amino acid including proline; each broken line, independently, represents a bond to an H or a bond to one said R group, and each H' represents said bond or a hydrogen; p is an integer between 0 and 4 inclusive;

or Group I has the structure:



where n is between 0 and 3 inclusive,

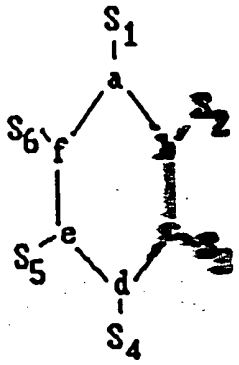
each G2 and G3 independently is H or C1 - 3 alkyl,

G1 is NH3, NH - C - NH2 ,or
                ‖
                NH2

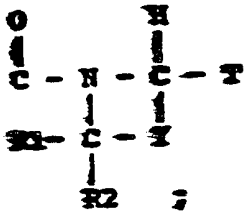
NG4, where G4 is C - G5
                |
                G6

where G5 and G6 can be NH, H, or C1 - 3 alkyl or alkenyl with one or more carbons substituted with a nitrogen; provided that G1 bears a charge and G1 and Group II do not form a covalently bonded ring structure at pH 7.0;

or Group I has the structure:

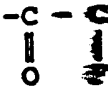


where one or two of said a, b, c, d, e, and f is N and the rest are C, and each S1 - S6 independently is H or C1 - C3 alkyl; where Group II has the structure:

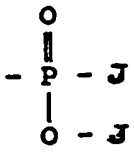


T is a group of the formula:

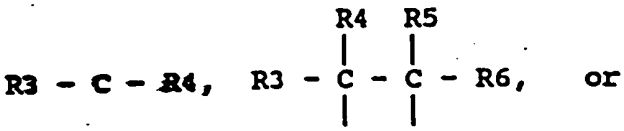
- B- D1, where B is boron and each D1 and D2, independently, is a hydroxyl group or a group which is capable of being hydrolysed to a hydroxyl group in aqueous solution at physiological pH; a group of the formula:

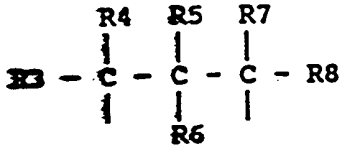


where G is either H, F or an alkyl group containing 1 to 20 carbon atoms and optional heteroatoms which can be N, S, or O; or a phosphonate group of the formula:



where each J, independently, is O-alkyl, N-alkyl, or alkyl, each said O-alkyl, N-alkyl or alkyl comprising 1 - 20 carbon atoms and, optionally, heteroatoms which can be N, S, or O; said T being able to form a complex with the catalytic site of a dipeptidyl-aminopeptidase type IV (DP IV) enzyme;

Y is  or



and each R1, R2, R3, R4, R5, R6, R7, and R8, separately is a group which does not significantly interfere with site specific recognition of said inhibitory compound by said DP

IV, and allows said complex to be formed with said DP IV for the preparation of a medicament for the treatment of transplant rejection or an autoimmune disease.

2. The use of claim 1, wherein T is a boronate group.

3. The use of claim 1, wherein T is a phosphonate group or a trifluoroalkyl ketone group.

4. The use of claim 1 wherein each R1 - R8 is H.

5. The use of claim 1 or 2 wherein each R1 and R2 are H, and each Y is CH_2 - CH_2.

6. The use of claim 5 wherein each R is independently chosen from the R group of proline and alanine.

7. The use of claim 1, wherein said compound has a binding or dissociation constant to said DP IV of at least 10^{-9}M.

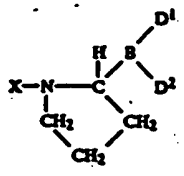
8. The use of claim 1, wherein said compound has a binding constant to said DP IV of at least 10^{-8}M.

9. The use of claim 1 admixed within a pharmaceutically acceptable carrier substance.

10. The use of claim 1 wherein, each D1 and D2 is, independently, F or D1 and D2 together are a ring containing 1 to about 20 carbon atoms, and optionally heteroatoms which can be N, S, or 0.

11. The use of claim 1, wherein said autoimmune disease is arthritis or systemic lupus erythmatosus.

12. The use of claim 1 wherein said compound has the formula



where each D^1 and D^2, independently, is a hydroxyl group or a group which is capable of being hydrolysed to a hydroxyl group in aqueous solution at physiological pH;

and X comprises an amino acid or a peptide which mimics the site of a substrate recognized by a post prolyl cleaving enzyme.